Effects of Surotomycin on *Clostridium difficile* Viability and Toxin Production *In Vitro*

Laurent Bouillaut[1], Shonna McBride[2,3], Joseph A. Sorg[4], Diane J. Schmidt[5], José M. Suarez[2#], Saul Tzipori[5], Carmela Mascio[6], Laurent Chesnel[6] and A. L. Sonenshein[1*]

[1] Department of Molecular Biology and Microbiology, Tufts University School of Medicine, Boston, MA USA; [2] Department of Microbiology and Immunology, and [3] Emory Antibiotic Resistance Center, Emory University School of Medicine, Atlanta, GA USA; [4] Department of Biology, Texas A&M University, College Station, TX USA; [5] Department of Infectious Disease and Global Health, Tufts University Cummings School of Veterinary Medicine, North Grafton, MA USA; [6] Cubist Pharmaceuticals, Lexington, MA USA

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* Corresponding author: A. L. Sonenshein, Department of Molecular Biology and Microbiology, Tufts University School of Medicine, 136 Harrison Avenue, Boston, MA 02111. Tel: 617-636-6761; Email: linc.sonenshein@tufts.edu

Current address: C/ Núñez de Balboa 54, 28001 Madrid, Spain

ABSTRACT

The increasing incidence and severity of infection by *Clostridium difficile* has stimulated attempts to develop new antimicrobial therapies. We report here the relative abilities of two antibiotics (metronidazole and vancomycin) in current use for treating *C. difficile* infection and a third antimicrobial, surotomycin, to kill *C. difficile* cells at various stages of development and to inhibit the production of the toxin proteins that are the major virulence factors. The results indicate that none of the drugs affects the viability of spores at 8X MIC or 80X MIC and that all of the drugs kill exponential phase cells when provided at 8X MIC. In contrast, none of the drugs killed stationary phase cells or inhibited toxin production when provided at 8X MIC and neither vancomycin nor metronidazole killed stationary phase cells when provided at 80X MIC. Surotomycin, on the other hand, did kill stationary phase cells when provided at 80X MIC, but did so without inducing lysis.

INTRODUCTION

In the United States, the incidence of *Clostridium difficile* infection (CDI) has been steadily rising over the last 15-20 years (1-3). There has been a significant increase in primary, recurrent and untreatable CDI on a global scale as well (3, 4). Treatment with oral vancomycin or metronidazole, the current standard practice, leads to recurrence of CDI in up to 30% of patients treated for an initial episode. For patients that have already suffered multiple recurrences, the future recurrence rate can be as high as 60% (3, 5-7).

46 In addition, many cases of CDI in the US are now linked to a family of more virulent
47 strains (NAP1/BI/027) that emerged first in the UK and then in Canada (8, 9).
48
49 *C. difficile* spores, the infectious form of the organism, are metabolically dormant but
50 germinate in response to a combination of host-derived bile acids and glycine (10).
51 Because spores are metabolically dormant, they are insensitive to the action of most
52 antibiotics. Thus, after antibiotics are prescribed to treat the active infection, the spores
53 that remain in the colon or that are acquired from the environment are able to germinate
54 and re-initiate active infection. The process of spore germination is largely enzymatic
55 and, once initiated, cannot be stopped. Once stimulated to germinate, the spore
56 releases a large depot of dipicolinic acid, chelated 1:1 with Ca^{++} (CaDPA), in exchange
57 for water (11). Also, the cortex layer, a specialized peptidoglycan, is degraded (12, 13).
58 After these changes, the spore is no longer dormant and does not have the resistances
59 typically characteristic of a spore. Metabolism then resumes within the spore core and
60 a vegetative cell grows out from the germinated spore. Such outgrowth is susceptible to
61 the action of several antibiotics (14). Moreover, the host intestine contains an
62 assortment of antimicrobial compounds that are produced by the innate immune system
63 and the host microbiota. To combat these defenses, *C. difficile* can modify its cell wall to
64 resist antimicrobials or efflux a variety of antimicrobial peptides from the cell (15-17).
65 These resistance mechanisms allow *C. difficile* to grow in the presence of antimicrobial
66 peptides and may also contribute to resistance to therapeutic antimicrobials.
67

Given the difficulties in treating *C. difficile* infections, the identification of narrow-spectrum antibiotics that are potent inhibitors of *C. difficile* growth would fulfill a critical need. Also needed are compounds or methods of reducing spore abundance or viability in order to limit the onset of infection and to prevent relapsing disease. Here we investigated the activities of a newly-developed antibiotic, surotomycin (18, 19), and compared its activity to that of metronidazole and vancomycin, two antibiotics that are currently used for treatment of *C. difficile* infections. Surotomycin is a lipopeptide antibiotic that acts through depolarization of the membrane, leading to the loss of a proton gradient and cell death (19). The mechanism of action of surotomycin differs significantly from that of vancomycin (cell wall synthesis-inhibiting) and metronidazole (inhibition of cellular enzymatic functions), suggesting that surotomycin may affect *C. difficile* physiology differently than do these other antibiotics. We report here the relative sensitivities of *C. difficile* strains to surotomycin, metronidazole and vancomycin at multiple stages of the life cycle, i.e., as dormant (phase-bright) spores, as germinated (phase-dark) spores, as exponential phase cells and as stationary phase cells. In addition, we assessed the effects of the antibiotics on toxin gene expression and measured the effects of various mutations that affect the sensitivity of exponential phase cells to nisin (an antibiotic that creates holes in membranes and disrupts cell wall synthesis) on susceptibility to the three tested compounds.

MATERIALS AND METHODS

Strains used and growth conditions. *Clostridium difficile* strains UK1 (ribotype 027; provided by Dr. D. Gerding) (20) and JIR8094 (ribotype 012; provided by Dr. J. Rood) were routinely grown at 37ºC in brain heart infusion (BHI) medium supplemented with cysteine (0.1%) and yeast extract (0.5%), referred to here as BHIS, or in tryptose-yeast extract medium (3% tryptose, 2 % yeast extract, 0.1% thioglycollate), referred to as TY. All growth experiments were performed in Coy anaerobic chambers in an atmosphere containing 85% nitrogen, 10% hydrogen, and 5% carbon dioxide as previously described (21, 22). Susceptibility testing of surotomycin requires the presence of 50 mg (2.5 mEq) Ca^{++} per liter in the medium (23). The levels of Ca^{++} in the media used were determined by Laboratory Specialists Inc; following their instructions, the Ca^{++} concentration in all media was raised to 50 mg/L by addition of $CaCl_2{\cdot}2H_20$. Drugs were added where indicated. Surotomycin was provided by Cubist Pharmaceuticals. Metronidazole and vancomycin were purchased from Sigma-Aldrich. Nisin was obtained from MP Biomedicals.

The following mutant strains derived from JIR8094 were used in this study: MC112, a *lytC* (peptidoglycan hydrolase), nisin-tolerant mutant; MC119, a spontaneous *cprK* (nisin-resistant) mutant with increased resistance to lantibiotics and polymyxin B (16); MC120, a mutant with a TargeTron insertion in *dltD* resulting in increased susceptibility to nisin and polymyxin B (15); and, MC141, a mutant with a TargeTron insertion in *cprA* causing increased susceptibility to lantibiotics (16).

Minimal Inhibitory Concentrations. Minimal inhibitory concentrations (MICs) were determined by growth of bacteria in culture tubes or in 96-well plates (that had been pre-reduced for at least 24 hrs prior to use) in media (either BHIS or TY) supplemented with 2-fold serial dilutions of the test compounds. The initial bacterial titer was 5 x 10^5 colony-forming units (CFU) per ml. Each strain and each drug concentration was tested in duplicate in each MIC assay. The MIC was determined as the lowest concentration of drug at which no bacterial growth was detected after 18-24 hrs at 37°C. MIC assays were performed at least 3 times to ensure reproducibility of results.

Spore germination assay. Spores of *C. difficile* strain UK1 were produced and purified as described previously (14, 24, 25). Purified spores (1 x 10^7) were suspended in 1 mL BHIS medium or germination salts [0.3 mM $(NH_4)_2SO_4$, 6.6 mM KH_2PO_4, 15 mM NaCl, 59.5 mM $NaHCO_3$, and 35.2 mM Na_2HPO_4] supplemented with 10 mM glycine. Cultures were further supplemented with 2 mM taurocholic acid (TA) and the test compounds at 8X or 80X MIC. The initiation of spore germination was detected as the loss of optical density at 600 nm in a PerkinElmer Lambda 25 spectrophotometer.

Measurements of killing rates. For experiments involving phase-dark spores, 1 x 10^7 phase-bright spores of strain UK1 were first incubated for 10 minutes in BHIS medium supplemented with 2 mM TA. The suspension was then centrifuged for 1 min at 14,000 x g. The resulting pellet of phase-dark spores was suspended in BHIS medium at a concentration of 1 x 10^5 per ml with or without the test compounds at 8X MIC or 80X MIC in Teflon-coated tubes to prevent the adherence of spores to the tubes. Spores

were confirmed to be phase-dark by phase-contrast microscopy. For experiments using exponential phase vegetative cells, strains UK1 or JIR8094 (1 x 10^5 per ml) were suspended in BHIS medium supplemented with $CaCl_2$ with or without addition of the test compounds at 8X MIC for strain JIR8094 or at 8X MIC and 80X MIC for strain UK1. For stationary phase cell killing assays, strain UK1 was grown in TY medium for ~12 hours and then diluted in 30 ml TY supplemented with $CaCl_2$ to give an OD_{600}~0.1. After 12 hrs incubation at 37°C, at which point the cells had left exponential growth phase, the culture was subdivided and the test compounds were each added to a separate culture tube at 8X or 80X MIC. For all assays, samples were withdrawn at indicated time points thereafter and viability was scored by plating serial dilutions on BHIS agar without calcium or antibiotic. Plates were incubated in the anaerobic chamber overnight. Each killing rate assay was performed at least twice to ensure reproducibility of the results.

Measurements of toxin gene expression. An overnight culture of strain UK1 was diluted in 30 ml TY-Ca^{++} to give an OD_{600}~0.1. After 12 hrs incubation at 37°C, the culture was split and each test compound was added to a separate subculture at 8X MIC. (Toxin gene expression was not tested in cells exposed to drugs at 80X MIC because such treatment with surotomycin at that concentration led to cell death and prevented the isolation of intact mRNA.) RNA was prepared from cells harvested at 0, 2, 4, 8 and 24 hrs after addition of the drugs as previously described (26). RNA was quantified by absorbance using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific). Primers for qPCR were designed using the online PrimerQuest tool from Integrated DNA Technologies

(http://www.idtdna.com/Scitools/Applications/Primerquest). Synthesis of cDNA was performed on 500 ng of RNA using random hexamer primers and the QuantiTect® Reverse Transcription Kit (QIAgen) according to the manufacturer's recommendations. To control for chromosomal DNA contamination, mock cDNA synthesis reactions containing no reverse transcriptase were used as negative controls in subsequent amplifications. cDNA samples were diluted 4-fold and used as templates for qPCR of *rpoC* (primers oLB122 [CTAGCTGCTCCTATGTCTCACATC] and oLB123 [CCAGTCTCTCCTGGATCAACTA]) and *tcdA* (primers oLB131 [GTATGGATAGGTGGAGAAGTCA] and oLB132 [CTCTTCCTCTAGTAGCTGTAATGC]) using the Roche SYBR Green I PCR mix and a Roche LightCycler 480 II thermocycler. Reactions were performed in a final volume of 20 µl using 4 µl diluted cDNA and primers at 1 µM final concentration. Amplification included 45 cycles of the following steps: 10 s at 95 °C, 10 s at 52 °C, 15 s at 72 °C. Reactions were performed in triplicate using cDNA synthesized from each of a minimum of three biological replicates, and results are presented as the means and SEM of the data obtained. Results were calculated using the $2^{-\text{delta deltaCt}}$ method, in which the amount of target mRNA is normalized to that of an internal control transcript (*rpoC*) (27).

Measurements of toxin protein accumulation by ELISA. Culture supernatants of the samples used for RNA extraction (above) were assayed for toxin A levels by ELISA (28). In brief, 96-well plates were coated with antibody to toxin A (Novus Biologicals) overnight at 4°C. After washing with PBS containing 0.1% Tween 20, the plates were incubated with blocking agent and, after discarding the agent, incubated with culture

fluid samples with or without dilution for 1 hr at room temperature. After washing with PBS-Tween, the plates were incubated with secondary antibodies fused to HRP (Gallus Immunotech, Inc.). After 1 hr at room temperature, the plates were washed again with PBS-Tween and mixed with peroxidase substrate for 40 min at room temperature. The reactions were stopped with H_2SO_4 and the OD_{450} was determined using a plate reader. Standard curves were generated using purified recombinant toxin A (29).

RESULTS

Determining the MICs of the test compounds. The MICs for each of the test compounds were determined using exponential phase cells as the target. The MIC values varied according to the strain being tested and the growth medium used (**Table 1**). For all further experimentation, the concentration of each antibiotic used was based on the MIC of that antibiotic in the strain to be tested growing in the medium being used. The growth medium chosen for each experiment was based on previous experimental conditions.

MIC determination in mutant strains. A panel of mutant strains derived from JIR8094 and altered in susceptibility to nisin and other small, cationic antimicrobial peptides (CAMPs) was tested for their susceptibility to surotomycin, metronidazole and vancomycin. As shown in **Table 2**, all strains had identical MIC values for metronidazole, but the MIC values for surotomycin and vancomycin varied slightly. MC112 has a mutation in an autolysin that presumably alters the cell wall of the bacterium, resulting in

increased tolerance to nisin, though not a higher MIC value. (That is, MC112 survives exposure to higher concentrations of nisin than does the parent strain, but does not grow in the presence of nisin.) MC112 had similar growth to the wild-type parent in all antibiotics tested, including surotomycin, in which the strain grew at a 2-fold higher concentration.

MC119 carries a mutation in a regulatory protein that is known to control an ABC-transporter system (*cprABC*). As a result, the MC119 mutant has an extraordinarily high MIC value for growth in nisin. In a previous study, we determined that MC119 also is less susceptible to polymyxin B (16). The MC119 strain had a 2-fold higher MIC for both vancomycin and surotomycin. The strongly decreased susceptibility of MC119 to vancomycin and its modestly decreased susceptibility to surotomycin, and polymyxin B are consistent with the hypothesis that this mutant is altered in its response to antimicrobials that target the cell surface.

Effects of antibiotic exposure on the initiation of *C. difficile* UK1 spore germination. To test if surotomycin or vancomycin or metronidazole is able to affect *C. difficile* spore germination, purified spores of strain UK1 were incubated in BHIS-Ca^{++} with or without the germinant taurocholate (TA) (10) and with or without each of the antibiotics. The spores germinated, as measured by loss of OD_{600}, when suspended in BHIS-Ca^{++} supplemented with 2 mM TA, but not in the absence of TA (**Figure 1A – D**). (The loss of OD corresponds to the conversion of spores from a birefringent [phase-bright] form to a non-birefringent [phase-dark] form.) The addition of surotomycin or

metronidazole or vancomycin at 8x or 80x MIC had no effect, either positive or negative, on TA-dependent spore germination (**Figure 1A – D**). When similar experiments were performed in a buffer containing TA and glycine, a co-germinant, the same lack of effect of the antibiotics was seen (data not shown).

Effects of antibiotic exposure on germinated (phase-dark) spores. To determine the time after spore germination at which outgrowing vegetative cells become sensitive to the antibiotics, we added antibiotics to germinated (phase-dark) spores in BHIS-Ca^{++} and followed bacterial viability at intervals thereafter. When exposed to surotomycin, the outgrowing cells lost viability more rapidly than after exposure to the other drugs **(Table 3)**. Note that this experiment does not distinguish between the time needed for killing and the growth stage at which outgrowing vegetative cells become susceptible to the drugs.

Effects of surotomycin, metronidazole and vancomycin on exponential-phase cells. Strains UK1 and JIR8094 were grown at 37°C to early exponential phase in BHIS-Ca^{++}. After determining the initial titer, the cultures were divided and inoculated with the test drugs at concentrations corresponding to 8X or 80X MIC. Samples of each culture were taken at multiple time points and surviving cell titers were determined by serial dilution and plating on BHIS-Ca^{++} supplemented with taurocholic acid (TA). In exponential phase cells of strain UK1, surotomycin reduced viability by 90% more rapidly than did the other drugs **(Table 3)**. In exponential phase cells of strain JIR8094,

surotomycin, metronidazole and nisin were all rapid killers, whereas vancomycin reduced viability more slowly **(Fig. 2)**.

Effects of surotomycin, metronidazole and vancomycin on stationary phase cells. After incubation of a culture of strain UK1 in TY + $CaCl_2$ at 37°C for 12 hrs, the culture was split and each drug was added to a separate culture tube at 8X or 80X MIC. Samples were harvested at various intervals for CFU counts and OD_{600} readings. At 8X MIC, none of the three drugs reduced the turbidity of the culture (apparent cell mass) or viability substantially **(Figs. 3A and 4A)**. At 80X MIC, however, surotomycin greatly diminished viability (but not cell mass significantly, indicating killing without cell lysis), whereas vancomycin and metronidazole had no detectable impact on cell mass or viability **(Figs. 3B and Fig. 4B)**.

Effects of surotomycin, metronidazole and vancomycin on toxin gene expression and toxin release. To test the effects of the various drugs on toxin production, cells at an early stage of stationary phase were exposed to the individual compounds at 8X MIC. At intervals thereafter, samples were harvested and RNA was extracted for quantitation by qRT-PCR. As shown in **Fig. 5**, expression of *tcdA*, the gene that encodes toxin A, increased with time to similar extents in untreated cells and cells treated with drugs.

To measure toxin A release into the culture medium, the supernatant fluids of the samples used above for RNA extraction were assayed for toxin A levels by ELISA (see Materials and Methods). Standard curves were generated using purified toxin A. As

shown in **Fig 6**, the amount of toxin A in the culture fluid was not significantly altered compared to the no-drug control by exposure to surotomycin or metronidazole at 8x MIC at early stationary phase. Addition of vancomycin, however, reduced toxin release at 24 hrs.

DISCUSSION

The results presented here show that surotomycin kills cells that are growing out of germinated spores, exponential-phase cells and stationary-phase cells (in the latter case at 80X MIC only) of strain UK1 more rapidly than does metronidazole or vancomycin. Surotomycin is also a more rapid killer of exponential-phase cells of strain JIR8094 than is vancomycin. None of the antibiotics tested was able to inhibit or activate *C. difficile* spore germination. This result seems to fit with the mechanisms of action of the tested antibiotics. The initiation of spore germination, a mostly enzymatic process, would be unaffected by antibiotics that need to be metabolized before becoming potent (metronidazole) or by antibiotics that inhibit new cell wall synthesis (vancomycin). Surotomycin leads to loss of the proton gradient, but does so by depolarizing the membrane without causing permeability of molecules as large as 671 daltons (19). Such a mechanism might explain its lack of effect on the initiation of spore germination if the pores created are also not large enough to allow the escape of Ca^{++}-DPA from the spore core, a step necessary for the completion of germination (12, 13). Alternatively, surotomycin might be unable to penetrate the spore coat and cortex in order to gain access to the spore inner membrane.

295

296 Since outgrowing spores are metabolically active and synthesize new cell wall
297 peptidoglycan, it is not surprising that such spores are susceptible to all of the
298 antibiotics tested, although the heightened susceptibility of such cells to surotomycin
299 was not predictable. The greater activity of surotomycin may be due to its ability to
300 prevent the generation of a proton gradient needed for metabolic functions to resume.
301 After the loss of dormancy, metabolism resumes in the core. Subsequently, a
302 vegetative cell begins to grow from the germinated spore and this requires new cell wall
303 synthesis. Metronidazole, which requires cellular metabolism for activity (30), inhibited
304 growth in an intermediate time-frame with respect to surotomycin and vancomycin.
305 Because new cell wall synthesis occurs late during outgrowth, it is not surprising that
306 vancomycin required more time to inhibit growth than the other two antibiotics tested.

307

308 Although the ability of all three antibiotics to kill exponential phase cells was expected,
309 their differential effects on stationary phase cells suggest that surotomycin may be more
310 effective than vancomycin or metronidazole in reducing the severity and recurrence of
311 infection. That is, at 80X MIC, a relatively low dose compared to those used clinically,
312 surotomycin killed stationary phase cells rapidly. Since stationary phase cells are the
313 ones that form spores and produce toxins, a drug that kills both exponential and
314 stationary phase cells may be particularly effective in reducing the spore titer and toxin
315 levels in the colon and in stool.

316

317 The appearance of *C. difficile* mutants that have decreased susceptibility to surotomycin
318 is rare and the MIC shift is not more than 8-to-16 fold (18). Therefore, it is interesting
319 that certain mutant strains that were isolated on the basis of their increased tolerance or
320 resistance to nisin also showed slightly reduced susceptibility to surotomycin (**Table 2**).
321 The basis for this decreased sensitivity is not known, but is likely to reflect changes in
322 cell surface architecture. The decrease in susceptibility gained, however, would not be
323 expected to influence the effectiveness of surotomycin *in vivo*.

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420

FIGURE LEGENDS

Figure 1. Effect of antibiotic treatment on initiation of *C. difficile* spore germination.

C. difficile UK1 spores were suspended in BHIS-Ca^{++} medium (black) or medium supplemented with 8x MIC (A) or 80x MIC (B) surotomycin or 8x MIC (C) or 80x (D) vancomycin or metronidazole. Where indicated, the medium was supplemented with taurocholic acid (TA), an activator of germination. The initiation of spore germination was detected by measuring the loss of OD_{600}. Experiments were performed in duplicate; the presented data represent one such experiment. Germination plots from the two experiments were superimposable.

Figure 2. Effects of drugs at 8X MIC on survival of exponential phase cells of *C. difficile* strain JIR8094.

Exponential phase cultures (OD_{600} = 0.45) in BHIS-Ca^{++} were exposed to the three antibiotics and samples withdrawn at indicated times thereafter were assayed for viability (CFU/ml).

Figure 3. Effects of drugs on stationary phase cells of strain UK1 as measured by cell mass.

Panel A, each drug was added at 8X MIC to the cultures at early stationary phase; panel B, each drug was added at 80X MIC to the cultures at early stationary phase. At least 3 biological replicates were performed for each drug at each concentration.

Figure 4. Effects of drugs at 8X and 80X MIC on viability of strain UK1 stationary phase cells. Early stationary phase cells were exposed to antibiotics at 8X MIC (part A)

or 80X MIC (part B). At indicated time-points thereafter, samples were removed and plated for viable counts (CFU).

Figure 5. qRT-PCR analysis of *tcdA* expression following treatment with surotomycin, metronidazole or vancomycin at 8X MIC. Strain UK1 was grown in $CaCl_2$-supplemented TY medium for ~12 hrs as described above, at which time the culture was split and drugs were added at 8X MIC. At two, four, eight and twenty-four hrs after addition of drugs, cell samples were harvested, RNA was extracted, and cDNA was synthesized as described under Materials and Methods. cDNA corresponding to *tcdA* mRNA was quantified by real time-PCR. Reactions were performed in triplicate using cDNA synthesized from each of a minimum of three biological replicates, and results are presented as the means and SEM of the data obtained. Results were calculated using the $2^{-\text{delta deltaCt}}$ method, in which the amount of target mRNA was normalized to that of an internal control transcript (*rpoC*).

Figure 6. ELISA assays of toxin A in cultures of strain UK1 treated with surotomycin, metronidazole or vancomycin at 8X MIC. Culture fluids of cells exposed to antibiotics at early stationary phase were collected at the indicated times and assayed for toxin A by ELISA.

466 **Table 1. MIC values for antimicrobial compounds[a]**

467

	MIC (µg/ml)		
Strain (Growth Medium)	Surotomycin	Metronidazole	Vancomycin
UK1 (BHIS + Ca^{++})	1.5	0.5	0.3125
JIR8094 (BHIS + Ca^{++})	1	0.5	2
UK1 (TY + Ca^{++})	0.125	0.125	1
ATCC 70057 (TY + Ca^{++})	0.125-0.25	0.06-0.125	1

468

469 [a] The various strains were grown in the indicated media and assayed for susceptibility
470 to the indicated antibiotics provided in a series of two-fold dilutions. The MIC was
471 defined as the lowest concentration of antibiotic that prevented measurable growth.

472

473 **Table 2. MIC values for antimicrobial compounds on mutant strains[a]**

474

Strain	MIC (μg/ml)			
	Nisin	Surotomycin	Metronidazole	Vancomycin
JIR8094	180	1	0.5	2
MC112	180	2	0.5	2
MC119	1440	2	0.5	4
MC120	90	1	0.5	2
MC141	90	1	0.5	2

475 476 477 478 479 480 481 482

483 [a] Each strain and each drug concentration was tested in cells growing in BHIS medium
484 in duplicate for each assay. Assays were performed at least 3 times to ensure
485 reproducibility of results.

Table 3. Time required to reduce viability of *C. difficile* UK1 phase-dark spores and exponential-phase cells by 90%.

	Time to 90% Killing in Hours					
	8x MIC			80x MIC		
	Surotomycin	Metronidazole	Vancomycin	Surotomycin	Metronidazole	Vancomycin
UK1 phase-dark spores[a]	3.33	> 7	> 7	2	3	> 5
UK1 exponential-phase cells[b]	0.33	1.5	1.5	< 0.33	0.66	1.33

[a] Tubes containing BHIS medium supplemented with $CaCl_2$ and the indicated antibiotics (at 8X MIC or 80X MIC) were inoculated with phase-dark spores of strain UK1 and tested, after various times of incubation, for survival by plating on BHIS medium.

[b] Exponential-phase cells of strain UK1 growing in BHIS medium supplemented with $CaCl_2$ were exposed to two different concentrations (8X MIC and 80X MIC) of the indicated antibiotics. Survival was assayed by plating serial dilutions on BHIS-Ca^{++} medium.